



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|---|-----------|---|
| (51) International Patent Classification ⁴ : C12N 1/00, 9/00, 9/14 C12P 1/00, 1/04, 19/34 C07K 7/04, 15/04 | A1 | (11) International Publication Number: WO 87/ 04181 (43) International Publication Date: 16 July 1987 (16.07.87) |
| (21) International Application Number: PCT/US87/00044 (22) International Filing Date: 5 January 1987 (05.01.87) (31) Priority Application Numbers: 817,226 845,662 (32) Priority Dates: 8 January 1986 (08.01.86) 28 March 1986 (28.03.86) (33) Priority Country: US (71) Applicant (for all designated States except US): CAL- GENE, INC. [US/US]; 1920 Fifth Street, Davis, CA 95616 (US). (72) Inventor; and (75) Inventor/Applicant (for US only) : STALKER, David, M. [US/US]; 3317 Oyster Bay Avenue, Davis, CA 95616 (US). (74) Agent: PEPPER, Frederick, W.; Morgan & Finnegan, 345 Park Avenue, New York, NY 10154 (US). | | (81) Designated States: AT (European patent), AU, BE (Eu- ropean patent), BR, CH (European patent), DE (Eu- ropean patent), DK, FR (European patent), GB (Eu- ropean patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), RO, SE (European patent), SU, US. Published <i>With international search report.</i> |
| (54) Title: HALOARYLNITRILE DEGRADING GENE, ITS USE, AND CELLS CONTAINING THE SAME | | |
| (57) Abstract Nitrilase enzymes specific for the hydrolysis of the nitrile group of bromoxynil, nucleotide sequences encoding for such enzymes, and transformed cells in which the nitrilase expression is foreign. The transformed cells are capable of expressing the nitrilase enzyme to provide detoxification of an environment and protect bromoxynil-sensitive cells from its cytotoxic effect. Particularly, plants are developed which are resistant to bromoxynil. | | |

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|------------------------------|----|--|----|--------------------------|
| AT | Austria | FR | France | ML | Mali |
| AU | Australia | GA | Gabon | MR | Mauritania |
| BB | Barbados | GB | United Kingdom | MW | Malawi |
| BE | Belgium | HU | Hungary | NL | Netherlands |
| BG | Bulgaria | IT | Italy | NO | Norway |
| BJ | Benin | JP | Japan | RO | Romania |
| BR | Brazil | KP | Democratic People's Republic of Korea | SD | Sudan |
| CF | Central African Republic | KR | Republic of Korea | SE | Sweden |
| CG | Congo | LI | Liechtenstein | SN | Senegal |
| CH | Switzerland | LK | Sri Lanka | SU | Soviet Union |
| CM | Cameroon | LU | Luxembourg | TD | Chad |
| DE | Germany, Federal Republic of | MC | Monaco | TG | Togo |
| DK | Denmark | MG | Madagascar | US | United States of America |
| FI | Finland | | | | |

- 1 -

HALOARYLNITRILE DEGRADING GENE, ITS USE,
AND CELLS CONTAINING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application is a Continuation-in-part
of application Serial No. 845,662, filed March 28,
1986 which is a Continuation-in-part of application
Serial No. 817,226, filed January 8, 1986, which
incorporates both disclosures herein by reference.

10 BACKGROUND OF THE INVENTION

Field of the Invention

The opportunity to provide novel genetic
capabilities to microorganisms and cells of higher
organisms has opened up broad avenues to new
15 capabilities. In one arena is the concern with
various agents that are utilized for their cytotoxic
effect. For example, many compounds used in
agriculture are directed to the killing of pests,
weeds, or the like. In many cases, these compounds
20 can have a relatively long residence time or extended
residue.

In many situations, one wishes to
distinguish between species which are to be retained
and species which are to be killed. For example, it
25 is frequently desirable to selectively kill weeds
while having minimal adverse effect on crops. For
the most part, many of the broad spectrum herbicides
have significant adverse effect on the crop, so that
their use is primarily limited to preemergent use or
30 careful postemergent application.

- 2 -

It is therefore of great interest to be able to modify viable cells to make them resistant to stresses such as cytotoxic agents.

Description of the Relevant Literature

5 U.S. Patent No. 4,535,060 describes the use of a bacterial aroA gene to impart glyphosate resistance to glyphosate susceptible cells. Hsu and Camper, Can. J. Microbiol. (1976) 22:537-543, describe isolation of ioxynil degraders from
10 soil-enrichment cultures. Hsu and Clemson, Dissert. Abstr. Intrn. B36 (1976) No. 8, 3708, describe microbial degradation of a family of herbicides of 3,5-dihalogeno-4-hydroxybenzonitriles. Ingram and Pullin, Pestic. Sci. (1974) 5:287-291 describes the
15 persistence of bromoxynil in three soil types. Smith, Abstrp. Meeting Weed Soc. Am. (1971), pp. 16-17 describes the degradation of bromoxynil in Regina heavy clay. Smith and Fletcher, Hort. Res. (1964), 4:60-62, report on 3,5-dihalogeno-4-
20 hydroxybenzonitriles and soil microorganisms.

SUMMARY OF THE INVENTION

Nitrilases, nucleic acid sequences encoding such nitrilases, constructs containing the genes
25 coding such nitrilases under the transcriptional and translational regulatory control of regulatory genes recognized by a desired host to which the nitrilase genes are foreign, host cells containing such
constructs, and organisms and organism parts or
30 products containing such constructs are provided.

- 3 -

° The bromoxynil- and/or ioxynil-specific nitrilases find use for detoxifying habitats containing bromoxynil and related herbicides and protecting host cells from the cytotoxic effect of such herbicides. The constructs find use in distinguishing between
5 host cells containing the construct and host cells lacking such construct.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

10 In accordance with the subject invention, novel DNA sequences, constructs, transformed cells, plants, and peptides are provided relating to hydrolysis of halogenated hydroxybenzonitriles, particularly 3,5-dibromo- or 3,5-diiodo-4-hydroxybenzonitrile. The invention concerns the
15 production of an enzyme capable of hydrolyzing the nitrile so as to detoxify the herbicidal activity of the nitrile and provide protection to a cell or host sensitive to the herbicide or detoxify an environment contaminated with the herbicide.

20 The structural gene of interest may be obtained from a unicellular microorganism, particularly a bacterium, which is shown to be capable of employing the benzonitrile as a nitrogen source, usually being capable of employing the
25 benzonitrile as the exclusive nitrogen source. Hereafter, in referring to benzonitrile or a nitrilase, it is intended that the benzonitrile be a halogenated p-hydroxybenzonitrile, particularly 3,5-diiodo-or 3,5-dibromo-4-hydroxybenzonitrile, and
30 the nitrilase is a nitrilase which is capable of

- 4 -

° using such halogenated benzonitrile as a nitrogen source, particularly as its exclusive nitrogen source.

The enzyme can be obtained in different ways, conveniently from bacteria which exist naturally in an environment containing bromoxynil or ioxynil. Particularly, enteric bacteria, more
5 particularly of the species Klebsiella, are of interest. Klebsiella pneumoniae may be employed, more particularly var. ozaenae. Rather than isolation from soil, organisms may be grown in soil
10 or other medium at increasingly higher concentrations of the benzonitrile and reduced amounts of alternative nitrogen sources until organisms which survive employing the benzonitrile as the sole nitrogen source are obtained.

15 Regardless of the source of the bacterium containing the nitrilase, screening must be performed to insure that the nitrilase is efficient in the detoxification of the benzonitrile. In addition, the nitrilase should be specific for the benzonitrile
20 rather than other analogs, which lack the halogens, have other substituents, or the like. The nitrilase of this invention will therefore be specific for the benzonitriles, as defined, and be relatively inactive toward analogs or substantially less active toward
25 analogs. Desirably, there should be no significant reduction in rate of proliferation, that is, less than about 10% reduction, of the proliferation of the bacterium in the presence of a normal nitrogen source, e.g. ammonia, as compared to the benzonitrile
30 as the nitrogen source at comparable concentrations.

Such result will not be observed with non-specified benzonitriles.

Once one or more host strains have been identified, techniques may then be employed to identify the coding sequence for the nitrilase. The gene may be present on a chromosome or plasmid. The genome may be fragmented, particularly with a restriction endonuclease, where one or a multiple of endonucleases may be employed to provide fragments ranging from about 5kb to 50kb. These fragments may be cloned on appropriate vectors in a convenient bacterium, e.g., E. coli, and the resulting transformants screened for nitrilase activity, where the host organism provides a negative background.

Once one or more clones have been identified as having nitrilase activity, the extrachromosomal elements containing the desired DNA fragment, plasmids or viruses, may be isolated by conventional techniques, such as lysis of the host, precipitation of the DNA, and separation of the vector DNA, plasmid or virus DNA, from the chromosomal DNA. The extrachromosomal elements may then be cleaved by endonuclease restriction and the desired fragments isolated by various techniques for separation and identification of different sized fragments, e.g., electrophoresis, density gradient centrifugation, or the like.

Depending upon the size of the fragment, it will usually be further manipulated to reduce the size to more closely approximate the size of the gene and its flanking regulatory regions. Various

- 6 -

techniques exist for manipulating the fragment containing the sequence coding for the enzyme and its regulatory flanking sequences. Partial cleavage with different restriction enzymes in different reaction mixtures may be employed, followed by cloning of the fragments to determine which fragments still retain the ability to provide expression of the nitrilase.

Alternatively, the enzyme may be isolated and partially sequenced. Based on the amino acid sequence, probes can be prepared which may then be used to identify those fragments having the gene. By combining this approach with restriction enzyme cleavage, fragments can be cloned and screened for the presence of the desired gene. In addition, one may use exonucleases, such as Bal31 to remove nucleotides from one or both ends of the fragment to further reduce the number of superfluous nucleotides.

Alternatively, the gene may be cloned in an appropriate host and messenger RNA isolated by screening with a probe, by identification in an appropriate in vitro or in vivo translation system, e.g., *Xenopus* oocytes or reticulolysate, or the like. The isolated messenger may then be used for preparing cDNA using conventional techniques involving a reverse transcriptase and formation of the complementary chain with a DNA polymerase. In this instance, the resulting structural gene lacks the regulatory regions associated with transcription.

The DNA sequence containing the structural gene expressing the nitrilase may be joined to a wide variety of other DNA sequences for introduction into

an appropriate host cell. The companion sequence will depend upon the nature of the host, the manner of introduction of the DNA sequence into the host, and whether episomal maintenance or integration is desired.

For prokaryotic hosts, a wide variety of vectors exist which may be used for introduction by transformation, conjugation, transduction or transfection of the DNA sequence into a prokaryotic host. DNA sequences include a wide variety of plasmids, such as pBR322, pACYC184, pMB9, pRK290, etc.; cosmids, such as pVK100; or virus, such as P22, etc.

For eukaryotic hosts, a wide variety of techniques may be employed for DNA introduction into the host, such as transformation with Ca^{++} - precipitated DNA, involving a non-replicating DNA sequence, a plasmid or a minichromosome, transformation with a T-DNA containing sequence in Agrobacterium, microinjection with a micropipette, or electroporation. Depending upon whether a competent replication system is present in the DNA construction, will determine whether the DNA may be replicated as an episomal element, or the DNA may be integrated into the host genome, and the structural gene expressed in the host. Episomal elements may be employed, such as tumor inducing plasmids, e.g., Ti or Ri, or fragments thereof, or viruses, e.g., CaMV, TMV or fragments thereof, which are not lethal to the host, and where the structural gene is present in such episomal elements in a manner allowing for

- 8 -

- expression of the structural gene. Particularly of interest are fragments having the replication function and lacking other functions such as oncogenesis, virulence, etc.

5 The fragments obtained from the nitrilase source may be cloned employing an appropriate cloning vector. Cloning can be carried out in an appropriate unicellular microorganism, e.g., a bacterium, such as E. coli. Desirably, one may use a cosmid, where
10 partial or complete digestion provides fragments having about the desired size. For example, the cosmid pVK100 may be partially digested with an appropriate restriction enzyme and ligated to fragments resulting from either partial or complete
15 digestion of a plasmid, chromosome, or fragment thereof. Packaging will insure that only fragments of the desired size will be packaged and transduced into the host organism.

The host organism may be selected for benzonitrile resistance. The recipient strains may
20 be modified to provide for appropriate genetic traits which allow for selection of transductants. In microorganisms, the transductants may be used for conjugation to other microorganisms, using a mobilizing plasmid as required. Various techniques
25 may be used for further reducing the size of the fragment containing the structural gene for the nitrilase. For example, the cosmid vector may be isolated, cleaved with a variety of restriction endonucleases, e.g., EcoRI, BglII, SmaI, etc., and
30 the resulting fragments cloned in an appropriate

- 9 -

vector, conveniently the cosmid vector previously used. Instead of a cosmid vector, a variety of cloning vectors are available of small size, such as pACYC177 and pACYC184. Thus, fragments of preferably less than about 5kb, usually less than about 4kb, and more preferably less than about 2kb, can be cloned and provide for benzonitrile resistance.

Desirably, the fragment will be about 1kb and less than about 5kb, preferably less than about 4kb, particularly at least about 1047bp, more particularly including flanking regions of at least about 1100bp, preferably less than about 1.5kb. Of particular interest, is a BglII-SmaI fragment from Klebsiella ozaenae, more particularly a PstI-HincII fragment of about 1210bp.

The nitrilase enzyme may be expressed by any convenient source, either prokaryotic or eukaryotic, including bacteria, yeast, filamentous fungus, plant cells, etc. Where secretion is not obtained, the enzyme may be isolated by lysing the cells and isolating the nitrilase according to known ways. Useful ways include chromatography, electrophoresis, affinity chromatography, and the like. Conveniently, bromoxynil may be conjugated through an appropriate functionality, e.g., the carboxyl group, to an insoluble support and used as a packing for the isolation of the nitrilase.

The nitrilase specific activity will be at least about 0.1 umol ammonia/min/mg protein, generally at least about 0.5 or higher under conditions as described by Harper, Biochem. J. (1977)

- 10 -

° 167:685-692.

The purified enzyme can be used in a wide variety of ways. It may be used directly in assays for bromoxynil, ioxynil, or other related benzonitriles. Alternatively, the subject enzyme can
5 find use as a label in diagnostic assays, by being conjugated to an analyte of interest, e.g., a hapten or antigen, or to an antibody, as such assays are described in U.S. Patent Nos. 3,654,090; 3,817,837; and 3,850,752. The methods of conjugation, as well
10 as the determination of the concentration of an analyte are described in extensive detail in these patents, and the appropriate portions of their disclosures are incorporated herein by reference.

The DNA sequence encoding for the nitrilase
15 may be used in a variety of ways. The DNA sequence may be used as a probe for isolation of wild type or mutated nitrilases. Alternatively, the DNA sequence may be used for integration by recombination into a host to provide for imparting benzonitrile resistance
20 to the host.

With plant cells, the structural gene as part of a construction may be introduced into a plant cell nucleus by micropipette injection for
integration by recombination into the host genome.
25 Alternatively, electroporation may be employed into which the structural gene may be introduced for introduction into a plant host. Where the structural gene has been obtained from a source having regulatory signals which are not recognized by the
30 plant host, it may be necessary to introduce the

appropriate regulatory signals for expression. Where a virus or plasmid, e.g. tumor inducing plasmid, is employed and has been mapped, a restriction site can be chosen which is downstream from a promoter into which the structural gene may be inserted at the appropriate distance from the promoter. Where the DNA sequences do not provide an appropriate restriction site, one can digest for various times with an exonuclease, such as Bal31 and insert a synthetic restriction endonuclease site (linker).

Of particular interest is the use of a tumor-inducing plasmid, e.g., Ti or Ri, where the nitrilase gene may be integrated into plant cell chromosomes. Descriptions of the use of Ti-plasmids and Ri-plasmids may be found in PCT Publication Nos. WO84/02913, 02919 and 02920 and EPO Application 0 116 718, and Matzke and Chilton, J. Mol. App. Genetics (1981) 1:39-49.

By employing the T-DNA right border, or both borders, where the borders flank an expression cassette comprising the nitrilase structural gene under transcriptional and translational regulatory signals for initiation and termination recognized by the plant host, the expression cassette may be integrated into the plant genome and provide for expression of the nitrilase enzyme in the plant cell at various stages of differentiation.

Various constructs can be prepared providing for expression in plant cells. The constructs provide an expression cassette which is functional in plants for expression of the nitrilase in the plant

- 12 -

• host.

To provide for transcription, a variety of transcriptional initiation regions (promoter regions), either constitutive or inducible, may be employed.

5 The transcriptional initiation region is joined to the structural gene encoding the nitrilase to provide for transcriptional initiation upstream from the initiation codon, normally within about 200 bases of the initiation codon, where the untranslated
10 5'-region lacks an ATG.

 The 3'-end of the structural gene will have one or more stop codons which will be joined to a transcriptional termination region functional in a plant host, which termination region may be
15 associated with the same or different structural gene as the initiation region.

 The expression cassette is characterized by having in the direction of transcription the initiation region, the structural gene under the
20 transcriptional control of the initiation region, and the termination region providing for termination of transcription and processing of the messenger RNA, as appropriate.

 As transcriptional and translational
25 regulatory regions, conveniently opine promoter and terminator regions may be employed, which allow for constitutive expression of the nitrilase gene. Alternatively, other promoters and/or terminators may be employed, particularly promoters which provide for
30 inducible expression or regulated expression in a

plant host. Promoter regions which may be used from the Ti-plasmid include opine promoters, such as the octopine synthase promoter, nopaline synthase promoter, agropine synthase promoter, mannopine synthase promoter, or the like. Other promoters include viral promoters, such as CaMV Region VI promoter or full length (35S) promoter, the promoters associated with the ribulose-1,5-bisphosphate carboxylate genes, e.g., the small subunit, genes associated with phaseolin, protein storage, B-conglycinin, cellulose formation, or the like.

The various sequences may be joined together in conventional ways. The promoter region may be identified by the region being 5' from the structural gene, for example, the opine gene, and by restriction mapping and sequencing may be selected and isolated. Similarly, the terminator region may be isolated as the region 3' from the structural gene. The sequences may be cloned and joined in the proper orientation to provide for constitutive expression of the nitrilase gene in a plant host.

By modifying crop plant cells by introduction of a functional gene expressing the nitrilase enzyme, one can use bromoxynil, ioxynil, or analogous herbicide with a wide variety of crops at concentrations which ensure the substantially complete or complete removal of weeds, while leaving the crop relatively unaffected. In this manner, substantial economies can be achieved in that fertilizers and water may be more efficiently utilized, and the detrimental effects resulting from

- 14 -

the presence of weeds avoided.

The expression cassette expressing the nitrilase enzyme may be introduced into a wide variety of plants, both monocotyledon and dicotyledon, including maize, wheat, soybean, tobacco, cotton, tomatoes, potatoes, Brassica species, rice, peanuts, petunia, sunflower, sugar beet, turfgrass, etc. The gene may be present in cells or plant parts including callus, tissue, roots, tubers, propagules, plantlets, seed, leaves, seedlings, pollen, or the like.

By providing for benzonitrile-resistant plants, a wide variety of formulations may be employed for protecting crops from weeds, so as to enhance crop growth and reduce competition for nutrients. For example, bromoxynil could be used by itself for postemergence control of weeds with safened crops, such as sunflower, soybeans, corn, cotton, etc., or alternatively, in combination formulations with other products.

Conventional amounts of the pesticides would be applied to fields in the formulations to deliver from about 0.1 to 4 lb/acre, preferably 0.2 to 2 lb/acre, of bromoxynil, where the other herbicide would be in amounts to deliver from about 0.1 to 4 lb/acre of active ingredient. Formulations would include other additives, such as detergents, adjuvants, spreading agents, sticking agents, stabilizing agents, or the like. The formulations may either be wet or dry formulations, including flowable powders, emulsifiable concentrates and

- 15 -

liquid concentrates, as in known in the art.

The herbicidal solutions may be applied in accordance with conventional ways, for example, through spraying, irrigation, dusting, or the like.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Materials and Methods

Restriction enzymes and T4 ligase for ligations were utilized according to the manufacturer's recommendations. Standard methods in cloning and molecular analysis were performed according to Maniatis et al., (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. Clone analysis was performed as described by Ish-Horowitz et al., Nucl. Acids Res. (1981) 9:2989-2998.

E. coli strain MM294 was used for all cloning experiments. (Hanahan, Mol. Biol. (1983) 166:557-80.)

The levels of antibiotics when employed were: Cm (chloramphenicol) 25ug/ml; Tc (tetracycline) 10ug/ml; Ap (penicillin) 300ug/ml.

Transformations of plasmid DNAs in E. coli were performed according to Mandel and Higa, J. Mol. Biol. (1970) 53:159-162.

Bacterial isolates from a bromoxynil contaminated soil sample were isolated and screened. One such organism was identified as Klebsiella pneumoniae sub-species ozaenae. Partial purification

- 16 -

and characterization of the bromoxynil specific and nitrilase from the above organism yielded an active enzyme with an apparent molecular weight of 34kDal.

Upon repeated subculturing of K. ozaenae on solid L- agar, a variant was isolated which no longer was able to utilize bromoxynil as a sole nitrogen source when this variant organism was grown in defined liquid medium containing per liter KH_2PO_4 (1.5g), K_2HPO_4 (3.5g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1g), yeast extract (50mg), citrate, glycerol and succinate at 0.1%, and trace elements as described by Barnett and Ingraham, J. Appl. Bacteriol. (1975) 18:131-143. This medium henceforth will be known as YETE multi-carbon medium. The YETE multicarbon medium contained 0.05% bromoxynil. Although this organism did not utilize bromoxynil as sole nitrogen source, it would grow to full density in L-broth containing 0.05% bromoxynil. A K. ozaenae variant colony was selected and grown in 10mls of L-broth. Three independent K. ozaenae colonies were also chosen from a LB plate containing bromoxynil and grown under the same conditions. These same four K. ozaenae colonies were simultaneously grown in 10mls L-broth supplemented with 0.05% bromoxynil. Cultures were grown to full density to 30°C and mini-prep plasmid DNA prepared from each culture by the method of Ish-Horowitz et al., Nucl. Acids Res. (1981) 9:2989. Undigested plasmid DNAs were electrophoresed on a 0.5% agarose gel and the plasmid bands visualized by ethidium bromide staining.

The K. ozaenae variant organism revealed a

single plasmid species (68Kb in size) grown either in the presence or absence of bromoxynil. The three K. ozaenae colonies showed a larger plasmid species (90Kb) when grown in the presence of 0.05% bromoxynil. In the absence of bromoxynil, both plasmid forms are present in two of the three K. ozaenae colonies. This data indicates conversion of the larger plasmid species to a smaller form with the concomitant loss of approximately 22Kb of plasmid DNA when bromoxynil selection is relieved.

All four colonies were grown in 200mls L-broth containing 0.05% bromoxynil. Cells were disrupted with a French press, the high speed supernatants dialyzed against buffer containing 0.05M KPO, pH7.5; 2.5mM dithiothreitol (DTT) and the individual crude extracts assayed for bromoxynil specific nitrilase activity. A crude extract prepared from the K. ozaenae variant contained no detectable nitrilase activity while the other K. ozaenae crude extracts exhibited nitrilase specific activities of 0.124, 0.105 and 0.143umole NH₃/min/mg protein respectively. Cells (200ml) were grown at 30°C to mid log phase in M9 medium (Miller (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory) containing 0.1% glucose and 0.04% bromoxynil. Crude extracts were prepared by cell disruption, ultracentrifugation and dialysis of the supernatant in buffer containing 0.05M KPO₄ pH 7.5 and 2.5mM DTT. Substrate concentration was 3mM bromoxynil in all assays. Release of NH₃ was monitored according to Harper Biochem. J. (1977)

- 18 -

167:685-692. The ability of K. ozaenae variant to grow in L-broth containing bromoxynil may result in acquired impermeability of the organism to the compound. However, the organism cannot grow in defined media utilizing bromoxynil as sole nitrogen source.

5 In summary, the K. ozaenae nitrilase appears to be plasmid encoded. The gene(s) encoding the enzyme appears to reside on a 22Kb plasmid DNA segment spontaneously lost from the K. ozaenae plasmid in the absence of bromoxynil selection.
10 The K. ozaenae bromoxynil specific nitrilase is expressed in E. coli.

Plasmid DNA from K. ozaenae grown under 0.05% bromoxynil selection was prepared and the DNA transformed to E. coli strain MM294 (thi⁻, gyrA96, endI⁻, hsdR17). Transformants were selected on nitrogen deficient (N⁻) solid agarose minimal medium (containing per liter KH₂PO₄ (1.5g), K₂HPO₄ (3.5g), MgSO₄·7H₂O (0.1g) and 0.1% glucose) with the addition of 0.05% bromoxynil as sole nitrogen source. After 5 days incubation, 10 colonies appeared on the selective plates. These colonies were restreaked on L-agar plates containing 0.05% bromoxynil and tested for the presence of the thiamine auxotrophic marker in MM294. None of the colonies grew in minimal media in the absence of thiamine indicating the strain to be E. coli MM294. All colonies could grow in M9 medium supplemented with thiamine and 0.05% bromoxynil as sole nitrogen source. No growth was observed in this medium in the
30

- 19 -

absence of bromoxynil. Two of the colonies were selected for further analysis. When crude extract preparations of E. coli MM294 containing the 90kb plasmid were assayed for bromoxynil specific nitrilase activity, a specific activity of 0.216umole NH₃ released/min/mg was obtained. E. coli MM294 containing the smaller plasmid species produced no detectable nitrilase activity. The larger 90Kb plasmid in E. coli was designated pBrx1 while the smaller plasmid (68Kb) was designated pBrx1Δ.

To confirm that E. coli strain MM294 containing plasmid pBrx1 produces the proper metabolite as a result of a bromoxynil specific nitrilase reaction, a 2ml culture of MM294 (pBrx1) was grown for 24hr at 30°C in M9 medium supplemented with 0.05% bromoxynil. A culture filtrate sample was chromatographed on a C₁₈HPLC column. All input bromoxynil in the culture filtrate was converted to a new metabolite peak. The identity of the metabolite peak was determined by spectral analysis to be 3'5'-dibromo-4-hydroxybenzoic acid (DBHB). Thus, the product of the bromoxynil specific plasmid encoded nitrilase expression in E. coli is the same as that observed for K. ozaenae. The bromoxynil specific nitrilase gene is cloned in E. coli.

To determine whether the DNA segment encoding the bromoxynil specific enzyme is clonable in E. coli, plasmid pBrx1 was digested with BamHI resulting in two bands of 53Kb and 37Kb, respectively. The BamHI fragments were ligated into

- 20 -

the BamHI site of the E. coli plasmid vector pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141) and transformed to E. coli strain MM294. Cloning into the BamHI site of pACYC184 results in insertional inactivation of the tetracycline resistance gene.

5 Ten chloramphenicol resistant tetracycline sensitive MM294 colonies were selected, mini-prep clone analysis DNA prepared and the DNA digested with BamHI. Four clones contained the 37Kb BamHI fragment while one clone harbored the larger 53Kb BamHI DNA

10 fragment of pBrx1. Five clones contained a cloned BamHI fragment also found in plasmid pBrx1 Δ which corresponds to the DNA segment remaining after spontaneous deletion of 22Kb of plasmid DNA from pBrx1. All 10 clones were grown in 200ml L-broth in

15 the presence of 20ug/ml chloramphenicol (to select for the plasmid), crude extract preparations obtained and assayed for bromoxynil specific nitrilase activity. Four clones containing the 37Kb BamHI fragment exhibited nitrilase specific activities in

20 the range of 0.140umole NH₃ released/min/mg protein while no detectable nitrilase activity was observed in the other six clones. This data indicates the gene encoding a bromoxynil specific nitrilase activity is located on a 37Kb BamHI fragment cloned

25 from plasmid pBrx1 and that the 22Kb DNA segment spontaneously lost in the absence of bromoxynil selection is internal to the 37Kb BamHI fragment.

To confirm the orientation of the BamHI fragments with respect to the vector pACYC184, DNA

30 from the above four clones was digested with ECORI

- 21 -

and electrophoresed on a 0.07% agarose gel. A combined EcoRI digest of plasmids pBrx1 and pBrx1 Δ was also analyzed.

Both orientations of the 37Kb BamH7 fragment with respect to the vector pACYC184 were defined and designated plasmids pBrx2 and pBrx3, respectively. It was also observed that the three EcoRI fragments are internal to the 22Kb DNA segment that is spontaneously deleted from plasmids pBrx2 and pBrx3. The sizes of these EcoRI fragments are 18Kb, 3Kb and 1.9Kb, respectively. The gene encoding the bromoxynil specific nitrilase should be located within one of these three EcoRI fragments if the nitrilase structural gene is not bisected by an EcoRI restriction site.

Localization of the bromoxynil specific nitrilase of E. coli (pBrx3) was investigated. The results were as follows.

- 22 -

TABLE 1

The Bromoxynil Specific Nitrilase is a
Periplasmic Enzyme in E. coli.

| 5 | <u>Culture Conditions^a</u> | <u>Nitrilase Specific Activity^b</u> |
|----|---------------------------------------|--|
| | toluenized cells (L-broth) | 0.829 |
| | lysozyme treated cells (L-broth) | 0.796 |
| | whole cells (L-broth) | 0.770 |
| 10 | whole cells (L-broth + Brx1) | 1.25 |
| | whole cells (M9) | 0.950 |
| | whole cells (M9 + Brx1) | 1.45 |
| | whole cells/pACYC184 (M9) | 0 |

15

a E. coli (MM294) cells containing plasmid pBrx3 were grown to stationary phase in 5ml cultures at 37° in medium indicated. Cultures contained 20ug/ml chloramphenicol and 0.04% bromoxynil (Brx1) where indicated. One ml from each culture was harvested, washed once with nitrilase buffer (0.1M KPO₄ pH7.5) and cells resuspended in 0.1ml of this same buffer. 50ul samples were assayed for nitrilase activity according to Harper, Biochem. J. (1977) 167:685-692, with and without 3mM bromoxynil as substrate.

20

b umole NH₃/min/mg. Protein was determined as O.D.600 of 1.4 = 10⁹ cells/ml = 150ug.

25

These data indicate that the cellular location of the nitrilase enzyme is the periplasmic space. A second observation is that the enzyme is expressed in the

30

- 23 -

absence of bromoxynil in the medium suggesting that bromoxynil induction is not required for enzyme expression.

Further purification of the bromoxynil specific nitrilase.

Further purification of K. ozaenae nitrilase was carried out with the following results.

TABLE 2

Purification from E. coli of the
Bromoxynil Specific Nitrilase.
(Starting material 6gms cells)

| <u>Fraction</u> | <u>Volume</u> | <u>Protein</u> | <u>umole NH₃/min</u> | <u>S.A.^b</u> |
|---|---------------|----------------|---------------------------------|-------------------------|
| Crude ^a | 100ml | 210mg | 18.15 | 0.086 |
| 35-50% NH ₄ SO ₄ | 6ml | 83mg | 26.77 | 0.250 |
| DEAE Sephadex | 56ml | 19mg | 15.52 | 0.820 |

^a Cells were grown at 30° to mid log phase in M9 medium containing 0.04% bromoxynil and glucose. Crude extracts were prepared by cell disruption, ultracentrifugation and dialysis in buffer containing 0.05M KPO₄ pH7.5 and 2.5mM DTT. Substrate concentration was 3mM in all nitrilase assays.

^b umole NH₃/min/ng.

- 24 -

• A 2.5cm² x 10cm column was equilibrated in buffer containing 0.05% KPO₄ pH7.5, 2.5mM DTT and 1mM EDTA. The sample was applied and the column developed with a 300ml linear gradient of 0.02M to 0.40M NaCl in the above column buffer. Buffer containing 1M NaCl was applied at the end of the gradient. 5ml fractions were collected and 0.075ml aliquots of alternate fractions assayed for nitrilase activity. A single peak of enzyme activity eluted at 0.22M salt. Approximately 75% of the input nitrilase activity was recovered in the active fractions.

Fractions spanning the nitrilase peak from the DEAE column were dialyzed against 0.02M KPO₄ pH7.5 and 50ul (6ug protein) of each fraction applied to an 11.25% denaturing Laemmli gel. The enriched protein band that corresponds to the activity peak from the DEAE column is a polypeptide of 34,000 molecular weight. No other polypeptides were enriched by the active column fractions. These data support that the bromoxynil specific nitrilase is a polypeptide of approximately 34,000 molecular weight and probably the product of a single gene.

Clone pBrx2 was completely digested with EcoRI and an approximately 19kb fragment isolated. The fragment was inserted into the EcoRI-digested pACYC184 vector (3.9kb) to provide the plasmid pBrx5 which was transformed into E. coli as described previously. The plasmid was isolated in conventional ways and digested with BglII to provide an approximately 6.7kb fragment which remained inserted in the pACYC184 vector. The isolated plasmid pBrx7

was then digested with SmaI and BglII to provide an approximately 3.9kb fragment which was inserted into SmaI-BamHI digested pACYC177 (3.7kb) (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156). The resulting plasmid which provided penicillin resistance was transformed into E. coli as described previously and transformants selected on penicillin selected medium to provide plasmid pBrx8, which carries the nitrilase gene on a 3.9kb fragment.

pBrx8 is partially digested with PstI and the fragments inserted into PstI digested pUC18 (Yanisch-Perron et al., Gene (1985) 33:103-119). The resulting plasmids were cloned in E. coli and screened for nitrilase activity. One clone had a 5.3kb plasmid pBrx9 which was isolated and further digested with PstI and HincII resulting in a 1210bp fragment having in the direction of PstI to HincII, ClaI, SalI, ScaI, and SphI restriction sites relatively evenly spaced. The PstI-HincII fragment was sequenced according to the method of Sanger et al., Proc. Natl. Acad. Sci. USA (1977) 74:5463-5468. The resulting sequence (with the appropriate amino acids encoded) is set forth in the following sequence.

25

30

65
 CTGCAGGATAGTAGGGCTTGAAGAGGATACGCTGTTTGGCGAGCCATCAAAATAAGGGGATTTTC
 125
 ATG GAC ACC ACT TTC AAA GCA GCC GCT GTT CAG GCC GAA CCG GTA TGG ATG GAT GCC GCT
 Met Asp Thr Thr Phe Lys Ala Ala Val Gln Ala Glu pro Val Trp Met Asp Ala Ala
 155
 GCA ACA GCC GAT AAG ACC GTG ACG CTA GTA GCT AAA GCC GCA GCG GCT GGC GCG CAG CTC
 Ala Thr Ala Asp Lys Thr Val Thr Leu Val Ala Lys Ala Ala Ala Gly Ala Gln Leu
 215
 GTC GCA TTT CCC GAA TTG TGG ATT CCG GGC TAC CCA GGA TTC ATG CTC ACG CAC AAC CAA
 Val Ala Phe pro Glu Leu Trp Ile pro Gly Try pro Gly Phe Met Leu Thr His Asn Gln
 245
 ACC GAA ACC CTA CCA TTC ATC ATT AAA TAC CGC AAG CAG GCA ATC GCC GCC GAT GGA CCA
 Thr Glu Thr Leu pro Phe Ile Ile Lys Try Arg Lys Gln Ala Ile Ala Ala Asp Gly pro
 305
 GAA ATC GAA AAA ATT CGC TGC GCG GCT CAG GAG CAT AAC ATT GCG CTC TCC TTT GGG TAC
 Glu Ile Glu Lys Ile Arg Cys Ala Ala Gln Glu His Asn Ile Ala Leu Ser Phe Gly Tyr
 335
 AGC GAA CGG GCT GGC CGT ACT CTC TAC ATG TCA CAA ATG CTT ATC GAT GCC GAT GGC ATC
 Ser Glu Arg Ala Glu Arg Thr Leu Tyr Met Ser Gln Met Leu Ile Asp Ala Asp Gly Ile
 395
 ACC AAA ATT CGT CGT CGA AAG CTC AAA CCA ACC CGC TTT GAA CGA GAA CTC TTT GGC GAA
 Thr Lys Ile Arg Arg Arg Lys Leu Lys pro Thr Arg Phe Glu Arg Glu Leu Phe Gly Glu
 455
 GGT GAC GGA TCG GAC TTA CAG GTC GCC CAA ACT AGC GTT GGT CGG GTG GGT GCC CTC AAC
 Gly Asp Gly Ser Asp Leu Gln Val Ala Gln Thr Ser Val Gly Arg Val Gly Ala Leu Asn
 515
 TGC GCG GAG AAT TTG CAG TCG CTA AAC AAC TTT GCG CTT GCT GCC GAG GGT GAA CAG ATA
 Cys Ala Glu Asn Leu Gln Ser Leu Asn Lys Phe Ala Leu Ala Ala Glu Gly Glu Gln Ile
 575
 605

- 27 -

655
 CAT ATC TCC GCC TGG CCA TTC ACG CTT GGA AGC CCT GTG CTC GTC GGA GAC TCC ATC GGC 665
 His Ile Ser Ala Trp Pro Phe Thr Leu Gly Ser Pro Val Leu Val Gly Asp Ser Ile Gly

 695
 GCC ATC AAC CAG GTC TAC GCG GCC GAG ACG GGG ACC TTC Phe Val Leu Met Ser Thr Gln Val 725
 Ala Ile Asn Gln Val Tyr Ala Ala Glu Thr Gly Thr Phe Val Leu Met Ser Thr Gln Val

 755
 GTT GGA CCG ACC GGC ATC GCC GCC TTC GAG ATC GAA GAC AGG TAC AAC CCG AAT CAG TAT 785
 Val Gly Pro Thr Gly Ile Ala Ala Phe Glu Ile Glu Asp Arg Tyr Asn Pro Asn Gln Tyr

 815
 CTT GGT GGT GGG TAC GCG CCG ATC TAC GGC CCT GAC ATG CAG TTG AAG AGC AAG TCG TTG 845
 Leu Gly Gly Gly Tyr Ala Arg Ile Tyr Gly Pro Asp Met Gln Leu Lys Ser Lys Ser Leu

 875
 TCA CCG ACC GAA GAG GGC ATC GTC TAC GCC GAG ATC GAC CTG TCG ATG CTT GAG GCA GCA 905
 Ser Pro Thr Glu Glu Gly Ile Val Tyr Ala Glu Ile Asp Leu Ser Met Leu Glu Ala Ala

 935
 AAG TAC TCG CTC GAT CCC ACG GGC CAC TAT TCG CGC CCT GAT GTG TTC AGC GTG TCG ATT 965
 Lys Tyr Ser Leu Asp Pro Thr Gly His Tyr Ser Arg Pro Asp Val Phe Ser Val Ser Ile

 995
 AAC CCG CAA CGG CAG CCT GCG GTG TCA GAA GTT ATC GAC TCA AAC GGT GAC GAG GAC CCG 1025
 Asn Arg Gln Arg Gln Pro Ala Val Ser Glu Val Ile Asp Ser Asn Gly Asp Glu Asp Pro

 1055
 AGA GCA GCA TGC GAG CCC GAC GAG GGG GAT CGT GAG GTC GTA ATC TCT ACG GCA ATA GGG 1085
 Arg Ala Ala Cys Glu Pro Asp Glu Gly Asp Arg Glu Val Val Ile Ser Thr Ala Ile Gly

 1115
 GTT CTA CCC CGT TAT TGC GGA CAT TCC TAATAAAAGAGACACACGCTGGTACCAAGGGGTTCATGTCCA 1155
 Val Leu Pro Arg Tyr Cys Gly His Ser

1200
 GACGCAGAAAATATAGCCCAGAGTTAAACGCGAAGCCATCGCTTTAAACCCGTC AAC

- 28 -

The PstI-HincII fragment substantially free of 5'- and 3'-non-coding flanking regions may be ligated with EcoRI linkers, digested with EcoRI and is now ready to be introduced into a plant expression cassette by insertion into the EcoRI site of pCGN451.

5 pCGN451 includes an octopine cassette which contains about 1,566bp of the 5' non-coding region fused via an EcoRI linker to the 3' end of the gene and about 1,349bp of 3' non-coding DNA. The pTi coordinates are 11,207 to 12,823 for the 3' region
10 and 13,643 to 15,208 at the 5' region as defined by Barker et al., Plant Molecular Biology (1983) 2:335. The 5' fragment was obtained as follows: A small subcloned fragment containing the 5' end of the coding region, as a BamHI-EcoRI fragment was cloned
15 in pBR322 as plasmid pCGN407. The BamHI-EcoRI fragment has an XmnI site in the coding region, while pBR322 has two XmnI sites. pCGN407 was digested with XmnI, resected with Bal31 nuclease and EcoRI linkers added to the fragments. After EcoRI and BamHI
20 digestion, the fragments were size fractionated, the fractions cloned and sequenced. In one case, the entire coding region and 10bp of the 5' non-translated sequences had been removed leaving the 5' non-transcribed region, the mRNA cap site and 16bp of the
25 5' non-translated region (to a BamHI site) intact. This small fragment was obtained by size fractionation on a 7% acrylamide gel and fragments approximately 130bp long eluted. This size fractionated DNA was ligated into M13mp9 and several
30 clones sequenced and the sequence compared to the

- 29 -

known sequence of the octopine synthase gene. The M13 construct was designated pI4, which plasmid was digested with BamHI and EcoRI to provide the small fragment which was ligated to an XhoI to BamHI fragment containing upstream 5' sequences from pTiA6 (Garfinkel and Nester, J. Bacteriol. (1980) 144:732) and to an EcoRI to XhoI fragment containing the 3' sequences. The resulting XhoI fragment was cloned into the XhoI site of a pUC8 derivative, designated pCGN426. This plasmid differs from pUC8 by having the sole EcoRI site filled in with DNA polymerase I, and having lost the PstI and HindIII site by nuclease contamination of HincII restriction endonuclease, when a XhoI linker was inserted into the unique HincII site of pUC8. The resulting plasmid pCGN451 has a single EcoRI site for the insertion of protein coding sequences between the 5' non-coding region (which contains 1,550bp of 5' non-transcribed sequence including the right border of the T-DNA, the mRNA cap site and 16bp of 5' non-translated sequence) and the 3' region (which contains 267 bp of the coding region, the stop codon, 196bp of 3' non-translated DNA, the polyA site and 1,153bp of 3' non-transcribed sequence).

The XhoI fragment containing the octopine synthetase (ocs) cassette was inserted into plasmid pCGN517, which has tetracycline resistance and kanamycin resistance genes. pCGN517 was prepared from pHC79 (Hohn, Gene (1980) 11:291) by introducing into the unique PstI site, the Kan^r gene from pUC4K (Vieira, Gene (1982) 19:259). pCGN517 was digested

- 30 -

with SalI and the XhoI fragment inserted into the unique SalI site.

The XhoI fragment was also inserted into a second plasmid pCGN529. pCGN529 is prepared from pACYC184 by insertion of the Kan^r gene from Tn5 (Rothstein et al., 1981, in Movable Genetic Elements, p. 99, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY) and a BglII fragment of 2.4kb from pRI4 T-LDNA (White and Nester, J. Bacteriol. (1980) 144:710) inserted into the BamHI site remaining after substitution of the HindIII-BamHI fragment of pACYC184 with the Kan^r gene of Tn5.

The XhoI fragment containing the ocs cassette into which the EcoRI nitrilase gene is inserted at the unique EcoRI of the ocs cassette is inserted into pCGN517 and pCGN529 to give two plasmids pN1 and pN2, respectively, which are used for introduction into A. tumefaciens or A. rhizogenes, respectively, for integration to the T-DNA of the Ti- or Ri-plasmids. Integration into the respective plasmids can be achieved in a 3-way mating as described by Comai et al., Plasmid (1983) 10:21-30. Overnight cultures of E. coli host containing plasmids pRK2073, pN1 or pN2 and A. tumefaciens A722 (Garfinkel, J. Bacteriol. (1980) 144:732) or A. rhizogenes A4T (White, ibid. (1980) 144:710) are cultured overnight and the appropriate cultures mixed and spread on AB plates containing 150ug/ml kanamycin. Single colonies are restreaked twice. Correct integration is verified by Southern analysis of total Agrobacterium DNA. Endonuclease

digested DNA is probed with nick-translated pBrx8.

The bromoxynil specific nitrilase gene is expressed in gall tissue.

5 The plasmid pBrx9, which carries the
nitrilase gene on a 2.6 kb fragment, was digested
with BamH1 and treated with Bal31 to remove some 5'
flanking region. BamH1 linkers were added and
reclosure was accomplished. The resulting plasmids
10 which provided ampicillin resistance were transformed
into E. coli as described previously and
transformants selected on ampicillin selective medium
to provide 5.2 kb plasmids pBrx16 and pBrx17, which
carry the nitrilase gene on a 2.6 kb fragment.
15 pBrx16 was digested with BamH1 and partially digested
with HincII resulting in the 1.2 kb nitrilase gene
fragment.

 The BamH1-HincII fragment was inserted into
BamH1-SmaI digested pCGN46 to provide the 6.6kb
20 plasmid pBrx22 containing the nitrilase gene fragment.

 PCGN46 (Comai et al., Nature (1985)
317:741-744) is a mannopine synthase (MAS) expression
casette and contains a MAS promoter and ocs 3'
region. Construction of pCGN46 was accomplished in
the following manner. An approximately 5.5kbp EcoRI
25 fragment (Eco13 or EcoC) carrying a portion of the
T-R DNA (Barker et al., Plant Mol. Biol. (1983)
2:325) including the mannopine synthase promoter
region (P_{MAS}) was cloned in a vector designated
30 pVK232. After digestion of pVK232 with EcoRI, Eco13

- 32 -

° was inserted into the EcoRI site of pACYC184 to yield plasmid pCGN14. pCGN14 was digested with SphI and ClaI (respectively at position 21562 and 20128 of the Barker et al. sequence, supra) to remove the P_{MAS} region which was inserted into pUC19 (Pharmacia, Inc.) which had been digested with SphI and AccI to yield pCGN40. The P_{MAS} region includes a ClaI recognition site internally which is methylated, so as to resist digestion.

10 pCGN40 was digested with ECORV and ECORI where the ECORV site is in the T-DNA, while the ECORI site is in the polylinker of pUC19 to provide a fragment having the P_{MAS} region. pCGN451 containing the octopine synthase cassette was digested with SmaI and ECORI and the larger fragment isolated from which the octopine synthase 5' region had been removed. The ECORV-ECORI P_{MAS} region was substituted into pCGN451 for the octopine synthase 5' region, where the transcriptional initiation and termination regions were separated by a polylinker to provide pCGN46.

20 The plasmid pBrx22 containing the 1.2 kb nitrilase gene fragment was transformed into E. coli as described previously. The plasmid was isolated in conventional ways and digested with Xho I to provide a 4.1 kb fragment containing MAS promoter, bromoxynil gene containing 25 base pairs of bacterial 5' untranslated sequence and ocs 3' region. The 4.1 kb fragment was inserted into the SalI-digested plasmid pCGN783 to provide the approximately 31 kb plasmid pBrx28.

Construction of pCGN783

Construction of pCGN167

To construct pCGN167, the AluI fragment of
CamV (bp 7144-7735) (Gardner et al. Nucl. Acids Res.
(1981) 9:2871-2888) was obtained by digestion with
5 AluI and cloned into the HincII site of M13mp7
(Vieira Gene (1982) 19:259) to create C614. An EcoRI
digest of C614 produced the EcoRI fragment from C614
containing the 35S promoter which was cloned into the
EcoRI site of PUC8 (Vierra et al., Gene (1982)
10 19:259) to produce pCGN146.

To trim the promoter region, the BglII site
(bp 7670) was treated with BglII and Bal31 and
subsequently a BglII linker was attached to the Bal31
treated DNA to produce pCGN147.

15 pCGN148a containing a promoter region,
selectable marker (KAN with 2 ATG's) and 3' region
was prepared by digesting pCGN528 (see below) with
BglII and inserting the BamHI-BglII promoter fragment
from pCGN147. This fragment was cloned into the
20 BglII site of pCGN528 so that the BglII site was
proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct,
pCGN528, was made as follows. pCGN525 was made by
digesting a plasmid containing Tn5 which harbors a
25 kanamycin gene (Jorgenson et al. Mol. Gen. (1979)
177:65) with HindIII-BamHI and inserting the
HindIII-BamHI fragment containing the kanamycin gene
into the HindIII-BamHI sites in the tetracycline gene
of pACYC184 (Chang & Cohen J. Bacteriol. (1978)
30 134,1141-1156). pCGN526 was made by inserting the

- 34 -

° BamHI fragment 19 of pTiA6 (Thomashow et al. Cell (1980) 19:729-739) into the BamHI site of pCGN525. pCGN528 was obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

5 pCGN149a was made by cloning the BamHI kanamycin gene fragment from pMB9KanXXI into the BamHI site of pCGN148a.

10 pMB9KanXXI is a pUC4K variant (Vieira & Messing, Gene (1982) 19:259:268) which has the XhoI site missing but contains a functional kanamycin gene from Tn903 to allow for efficient selection in Agrobacterium.

15 pCGN149a was digested with BglII and SphI. This small BglII-SphI fragment of pCGN149a was replaced with the BamHI-SphI fragment from M1 (see below) isolated by digestion with BamHI and SphI. This produces pCGN167, a construct containing a full length CaMV promoter, 1ATG-kanamycin gene, 3' end and the bacterial Tn903-type kanamycin gene. M1 is an EcoRI fragment from pCGN550 (see construction of pCGN587) and was cloned into the EcoRI cloning site of M13mp9 in such a way that the PstI site in the 1ATG-kanamycin gene was proximal to the polylinker region of M13mp9.

25 Construction of 709 (1ATG-Kanamycin - 3' region)

30 pCGN566 contains the EcoRI-HindIII linker of pUC18 (Yanisch-Perron, ibid) inserted into the EcoRI-HindIII sites of pUC13-cm (K. Buckley, Ph.D. thesis, UC-San Diego, 1985). The HindIII-BglII

- 35 -

fragment of pNW31c-8, 29-1 (Thomashow et al. (1980) Cell 19:729) containing ORF1 and 2 (Barker et al. (1983), supra) was subcloned into the HindIII-BamHI site of pCGN566 producing pCGN703.

The Sau3A fragment of pCGN703 containing the 3' region of transcript 7 from pTiA6 (corresponding to bases 2396-2920 of pT115955 (Barker et al. (1983), supra) was subcloned into the BamHI site of pUC18 (Yanisch-Perron et al. (1985), supra) producing pCGN709.

Construction of pCGN766c (35s promoter - 3' region)

The HindIII-BamHI fragment of pCGN167 (for construction see infra) containing the CaMV-35S promoter, 1ATG-kanamycin gene and the BamHI fragment 19 of pTiA6 was cloned into the BamHI-HindIII sites of pUC19 (Norrande et al. (1983), supra; Yanisch-Perron et al. (1985), supra) creating pCGN976.

The 35S promoter and 3' region from transcript 7 was developed by inserting a 0.7kb HindIII-EcoRI fragment of pCGN976 (35S promoter) and the 0.5kb EcoRI-SalI fragment of pCGN709 (transcript 7:3', for construction, see supra), into the HindIII-SalI sites of pCGN566 creating pCGN766c.

Final Construction of pCGN783

The 0.7kb HindIII-EcoRI fragment of pCGN766c (CaMV-35S promoter) was ligated to the 1.5kb EcoRI-SalI fragment of pCGN726c (1-ATG-KAN-3' region) into the HindIII-SalI sites of pUC119 (J. Vieira, Rutgers University, N.J.) to produce pCGN778.

The 2.2kb region of pCGN778, HindIII-SalI

- 36 -

fragment containing the CaMV 35S promoter (1-ATG-KAN-3' region) replaced the HindIII-SalI polylinker region of pCGN739 to produce pCGN783.

pBrx17 was digested with BamHI and partially digested with HincII resulting in the 1.2kb nitrilase gene fragment. The BamHI - HincII fragment was inserted into BamHI - SmaI digested pCGN566 to provide the 3.7kb plasmid pBrx25 containing the nitrilase gene fragment.

PCGN566 was constructed in the following manner. pUC13 (Cm^R) (Ken Buckley Ph.D. thesis, U.C., San Diego) was digested with EcoRI and HindIII and polylinkers from pUC18 and pUC19 were inserted respectively into the linearized pUC13 to give pCGN566 which carries a chloramphenicol resistance marker.

The plasmid pBrx25 containing the 1.2kb nitrilase gene fragment was transformed into E. coli as described previously. The plasmid was isolated in conventional ways and digested with BamHI and EcoRI to provide again the 1.2kb nitrilase gene fragment. The BamHI and EcoRI fragment was inserted into the BamHI and EcoRI digested pCGN46 to provide the 6.6kb plasmid pBrx27 containing the nitrilase gene fragment.

pBrx27 was transformed into E. coli as described previously. The plasmid was isolated in conventional ways and digested with XhoI to provide a 4.1kb fragment containing MAS promoter, bromoxynil gene containing 11 base pairs of bacterial 5' in translated sequence and ocs 3' region. The 4.1kb fragment was inserted into SAL I - digested pCGN783 to

provide the approximately 31kb plasmid pBrx29.

Detection of nitrilase expression

Plasmids pBrx28 and pBrx29 were transformed into the Agrobacterium tumefaciens strain K12.

(Nester, Ann. Rev. Micro. (1981) 35: 531. Hoekema et al., Nature (1983) 303: 179) K12 (pBrx28) and K12 (pBrx29) were used to form galls on Kalanchöe (Garfinkel, J. Bacteriol. (1980) 144: 732).

About 1gm (fresh weight) of gall tissue was ground in liquid nitrogen in buffer containing 0.1M Tris pH 7.5, 10mM EDTA, 0.15M NaCl, 0.05% NP-40, 25 mg/ml BSA, 1mM DTT and 0.13 ug/ml leupeptin. Samples were homogenized after the addition of 0.05g polyvinylpyrrolidone (Sigma), then centrifuged at 15,000g for 15 min. at 4°C. 25 ul of antiserum, prepared by injecting purified nitrilase into rabbits, and 250ul 10% (w/v) suspension of S. aureus (Calbiochem) were added to each supernatant and incubated for 16 hr. at 4°C. Samples were then centrifuged and the pellet washed twice with 20mM Tris pH 7.5, 1mM EDTA, 150mM NaCl and 0.05% NP-40. The pellets were resuspended in 100ul 0.125M Tris pH 6.8, 4% SDS, 20% glycerol and 10% BME and heated for 2 min. at 90°C. The entire sample was electrophoresed on 10% acrylamide gels (Laemmli, V.K. Nature 227: 680-685 (1970)). The resolved polypeptides were transferred to nitrocellulose filters (Schleicher and Schuell) as described by Burnette (Anal. Biochem. 112: 195-203 (1981)). Nitrocellulose filters (Schleicher & Schuell) were then incubated in BLOTTO (Johnson et al, Gen. Anal.

- 38 -

- Technol. 1, 38-42 (1983)) for 1-3 hrs. at 42°C. followed by overnight incubation at room temperature in BLOTTO containing a 1:50 solution of anti-nitrilase serum. Filters were washed for 10 min. in 20mM Tris pH 7.5, 150mM NaCl; for 20 min. in the same buffer containing 0.05% Tween-20 and for another 10 min. in buffer without Tween-20. BLOTTO containing 10^6 cpm/ml of 125 I-labelled protein A (9u Ci/mg; NEN) was then added to filters and incubated at room temperature for 2 hrs. The filters were washed overnight in 50mM Tris pH 7.5, 1M NaCl and 0.4% Sarkosyl. After rinsing and drying, filters were exposed to Kodak AR X-ray film at -70°C. using a Dupont Cronex intensifying serum.
- 15 Transformation and regeneration of tobacco leaf slices co-cultivated with A. rhizogenes

- Tobacco plants are cultivated axenically (25°C, white light (16hr); MS (1mg/L IAA, 0.15mg/L kinetin). Three-week-old plants maintained through main shoot transplant are used as tissue donors. Young leaves (down to the fourth from the top) are selected, leaf disks 2mm in diameter are punched out and placed in Petri dishes (3cm in diameter) in 1ml of MS medium with 1mg/L IAA. After keeping the disks overnight in total darkness, Agrobacterium (A772xpN1 or pN2) cells (10^8 - 10^9 /ml in plant culture medium) are added to these cultures. Co-cultivation is carried out for 18-24hr in darkness. Leaf slices are freed from Agrobacterium by washing 3x with MS medium lacking hormones and containing 350mg/L

- 39 -

0 cefotaxine (Boehringer-Mannheim). Leaf slices are transferred in 9cm Petri dishes in 10ml of MS medium without hormones. Phytagar (Gibco, 0.6%; cefotaxine, 350mg/L) Petri dishes are sealed with parafilm and kept under the same conditions as tissue donor
5 plants. Roots appear up to 2-4 weeks, are excised and placed under the same conditions in the same medium plus 2mg/L IAA and 2mg/L kinetin. Regenerating shoots are visible in the following 2-5 weeks.

10 Plants are sprayed at the 6-leaf stage by directing a spray of bromoxynil solution toward the potted plant. Each 4" pot contains a plant and receives 2.5ml of spray. Plants are grown in a growth chamber at 25°C, 70% relative humidity, 60hr light period. Growth is scored 9 days after spraying
15 by counting the new leaves longer than 0.5cm.

By following the above procedures, plants can be obtained which are bromoxynil resistant and can be used in the field in the presence of bromoxynil without significant adverse effect on
20 their growth.

The subject invention provides for the improvement of plants by making them herbicidal resistant, particularly to specific benzonitrile herbicides. Thus, the gene encoding for the
25 nitrilase may be introduced into a plant host, whereby the gene will be expressed and impart benzonitrile resistance to the plant. In addition, the enzyme can be produced by cloning of the gene in a convenient bacterial host, whereby the enzyme is
30 expressed. Enzymes having activity which can be

- 40 -

° monitored find a wide variety of uses, in assays for various analytes or for the benzonitrile substrate. In addition, the enzymes and bacteria expressing the enzymes can be used for removing the benzonitrile herbicide from contaminated environments.

5 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and
10 modifications may be practiced within the scope of the appended claims.

15

20

25

30

- 41 -

WHAT IS CLAIMED IS:

1. A substantially pure bacterial nitrilase of about 34kd at a purity having a specific activity of at least about 0.1 $\text{umol NH}_3/\text{min/mg}$ protein with bromoxynil as substrate.

2. A bacterial host having a foreign gene expressing a nitrilase specific for a 3,5-dihalogenated-p-hydroxybenzonitrile.

3. A composition comprising a nitrilase of about 34kDal, said composition having a specific activity of at least about 0.1 $\text{umol NH}_3/\text{min/mg}$ protein bromoxynil as substrate.

4. A composition according to Claim 3, wherein said nitrilase being a bacterial nitrilase.

5. A composition according to Claim 4, wherein said bacterial nitrilase is a nitrilase obtained from Klebsiella.

6. A composition according to Claim 3, wherein said composition has a specific activity of at least about 0.5 $\text{NH}_3/\text{min/mg}$ protein.

7. A bacterial host having a foreign gene expressing nitrilase specific for a 3,5-dihalogenated-p-hydroxybenzonitrile.

- 42 -

8. A bacterial host according to Claim 7, wherein said bacterial host is E. coli.

9. An expression cassette comprising a structural gene coding for a bromoxynil and/or ioxynil specific nitrilase under the transcriptional and translational regulatory control of regulatory regions functional in a plant cell.

10. An expression cassette according to Claim 9, wherein said nitrilase is a bacterial nitrilase.

11. An expression cassette according to Claim 9 or 10, wherein said cassette has at least one T-DNA border.

12. An expression cassette according to Claims 9 to 11, wherein said transcriptional initiation region is from a gene encoding an opine.

13. A plasmid capable of replication in at least one of E. coli and A. tumefaciens comprising an expression cassette according to Claim 9.

14. A plasmid according to Claim 13, wherein said expression cassette has at least one T-DNA border.

- 43 -

15. A DNA sequence substantially as set forth in the specification joined to and under the transcriptional regulatory region control of other than the wild type transcriptional initiation region for a bromoxynil and/or ioxynil specific bacterial nitrilase found in Klebsiella.

16. A DNA sequence according to Claim 15, wherein said transcriptional regulatory region is functional in a plant.

17. A DNA sequence according to Claim 15, wherein said transcriptional regulatory region is functional in a bacterium.

18. A DNA sequence having an open reading frame coding for a nitrilase enzyme specific for a 3,5-dihalogenated-p-hydroxybenzonitrile, said sequence having at its 5'-terminus other than the wild type transcriptional initiation region.

19. A DNA sequence according to Claim 18, wherein said nitrilase enzyme is a bacterial nitrilase enzyme.

20. A DNA sequence according to Claim 19, substantially homologous with a DNA sequence from Klebsiella.

21. A plant cell comprising an expression cassette according to Claims 9 to 12.

- 44 -

22. A plant containing a plant cell according to Claim 21.

23. A method for producing a nitrilase specific for a 3,5-dihalogenated-p-hydroxybenzonitrile which comprises:

isolating K. ozaenae which produce nitrilase specific for said 3,5-dihalogenated-p-hydroxybenzonitrile;

growing K. ozaenae in an appropriate nutrient medium; and

lysing said K. ozaenae and isolating said nitrilase.

24. A method for obtaining an enzyme having a selectable property which comprises:

screening bacteria for said selectable property and selecting said bacteria having said selectable property;

cleaving the genome of said bacteria to produce fragments of a desired size range;

cloning said fragments on appropriate vectors in a bacterium and selecting for enzymes having said selectable property; and

isolating the DNA sequence having the structural gene expressing said enzyme having said selectable property.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00044

| | | |
|---|---|---|
| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4) C12N 1/00, 9/00, 9/14; C12P 1/00; 1/04; 19/34 C07K 7/04; 15/04 U.S. 435/68, 240 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁴ | | |
| Classification System | Classification Symbols | |
| US | 435/68, 70, 91, 172.3, 240, 243, 253, 317 536/27; 935/56, 60, 72; 514/2; 530/350, 808, 825 | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶ | | |
| CHEMICAL ABSTRACTS DATA BASE (CAS) 1967-1987 BIOLOGICAL ABSTRACTS DATA BASE (BIOSIS) 1969-1987 Keywords: nitrilase, benzonitrilase, hydrobenzonitrilase, gene | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ | | |
| Category ⁸ | Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ |
| Y, P | <u>SCIENCE</u> (Washington, D.C., U.S.A.) Volume 233, issued 25 July, 1986, (SHAH ET AL), "Engineering Herbicide Tolerance in Transgenic Plants", see page 478. | 2-4, 6-24 |
| Y | <u>SCIENCE</u> (Washington, D.C., U.S.A.) Volume 221, issued 22 July, 1983, (COMAI ET AL), "An Altered <i>aroA</i> Gene Product Confers Resistance to the Herbicide Glyphosate", see page 370. | 2-4, 6-24 |
| Y | Chemical Abstracts, Volume 103, No. 11, issued 16 September 1985 (Columbus, Ohio, USA), HARPER, "Characterization of a nitrilase from <i>Nocardia</i> sp. (Rhodochrous group) N.C.I.B. 11215, using <i>p</i> -hydroxybenzonitrile as sole carbon source", see page 271, column 2, the abstract No. 83953g, <u>International Journal Biochemistry</u> 1985, 17(6), 677-683 (ENG). | 1,5 |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>⁹ Special categories of cited documents: ¹³</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search ² | | Date of Mailing of this International Search Report ² |
| 23 March 1987 | | 31 MAR 1987 |
| International Searching Authority ¹ | | Signature of Authorized Officer ³⁰ |
| ISA/US | | <i>Stephanie Seidman</i> Ph.D., J.D. Stephanie Seidman Ph.D., J.D. |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|--|-----|
| Y | Chemical Abstracts, Volume 102, No. 25, issued 24 June 1985 (Columbus, Ohio, USA), Yanase, "Metabolism of nitriles in Pseudomonas sp", see page 343, column 2 page 344, column 1, the abstract No. 218090a, <u>Journal Fermentation Technology</u> 1985, 63(2), 193-198 (ENG). | 1,5 |
|---|--|-----|

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹¹ | Relevant to Claim No. ¹² |
|------------|---|-------------------------------------|
| Y | Chemical Abstracts, Volume 102, No. 17, issued 29 April 1985 (Columbus, Ohio, USA), Stalker, "Impact of genetic engineering on crop protection", see page 329, column 1, the abstract No. 146080, <u>Crop Protection</u> 1984, 3(4), 399-408 (ENG). | 1-24 |
| Y, P | <u>JOURNAL OF CELLULAR BIOCHEMISTRY</u> (New York, New York, USA), Volume Supplement 10C, issued May, 1986, (STALKER ET AL), "Strategies Utilizing Bacterial Genes As Herbicide Resistance Determinants in Plants", see page 11. | 1-24 |
| Y, P | <u>JOURNAL OF CELLULAR BIOCHEMISTRY</u> (New York, New York, USA) Volume Supplement 10C, issued May, 1986, (CHALEFF ET AL), "Developing Plant Varieties Resistant to Sulfonylurea Herbicides", see page 10. | 1-24 |

This Page Blank (uspto)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)